

# Photoinhibition of marine nitrifying bacteria. I. Wavelength-dependent response

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**ABSTRACT:** Axenic cultures of chemolithotrophic nitrifying bacteria ( $\text{NH}_4^+$  and  $\text{NO}_2^-$  oxidizers) respond to light in distinct manners. After short-term (2 to 4 h) monochromatic irradiations both types of nitrifying bacteria demonstrated a widespread photosensitivity in the near-UV region (300 to 375 nm) and a differential photosensitivity in the blue region of the spectrum (400 to 475 nm). Nitrite oxidizers were less sensitive overall to blue light inhibition than were ammonium oxidizers. Characteristically, the extent of the photoinhibition was species-specific and light response was dose- and wavelength-dependent. Photoprotection by higher substrate concentrations was only observed with ammonium oxidizers. Increased cell densities altered the phototolerance of nitrite oxidizers and made these organisms light-susceptible. Similarly, treatment with a low light dose for extended periods was more damaging to nitrite oxidizers, when high cell densities were used. Polychromatic irradiations served to confirm the monochromatic results. Cool-white fluorescent light inhibited  $\text{NH}_4^+$  oxidizing activity but not  $\text{NO}_2^-$  oxidizing activity. Exposure to sunlight resulted in inhibition of activity in both types of nitrifiers. These data demonstrate that the effect of light on autotrophic nitrification depends not only on the type of nitrifier ( $\text{NH}_4^+$  or  $\text{NO}_2^-$  oxidizer), but also on the conditions of their environment.

**KEY WORDS:** Nitrification · Photoinhibition · Nitrifying bacteria

## INTRODUCTION

Nitrification constitutes the only biological mechanism for the conversion of  $\text{NH}_4^+$  to  $\text{NO}_3^-$ . Much of the earlier work on factors affecting nitrification ( $\text{NH}_4^+$  and  $\text{NO}_2^-$  oxidation) was done with soil nitrifying bacteria because of their agricultural importance (Focht & Verstraete 1977). These works established that light can regulate the process of nitrification as it can inhibit both ammonium and nitrite oxidizing activities (Müller-Neuglück & Engel 1961, Schön & Engel 1962). Light impact on nitrification gains importance in aquatic systems, since in these systems light penetration can occur to different depths (Jerlov 1968, Craig 1973, Gieskes et al. 1989) depending on the water transparency. Thus, along with the usual environmental limitations, such as substrate availability, pH,  $\text{pO}_2$ , and temperature, aquatic nitrifiers face the additional

stress of light. Thus far, there have been contrasting results as to which step of the process is more photosensitive. Olson (1981), Yoshioka & Saijo (1984), and Diab & Shilo (1988) have reported nitrite oxidation as being especially sensitive to light in oceanic, freshwater and aquaculture ponds, respectively. However, Vanzella et al. (1989) described ammonium oxidation as the step most affected by light using pure cultures and marine samples. Horrigan & Springer (1990) reported dissimilar photosensitivities in  $\text{NH}_4^+$  oxidizers from different habitats (estuarine and marine). Among the reasons that may have contributed to the apparent differences in results are the facts that some workers used natural populations as opposed to pure cultures, that different light sources and intensities were used, and that the species tested and incubation conditions used were different. For comparison purposes, the present work used 4 representative marine species to examine the photoresponse of both ammonium and nitrite oxidizers, under standardized conditions. In addition, variables such as substrate concentration,

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light intensity, light quality (wavelength), dose rate and cell density were considered, since these factors can vary naturally. Ecological consequences of the light sensitivity in this 2-step process are discussed.

## MATERIALS AND METHODS

**Microorganisms and growth conditions.** The marine ammonium oxidizing bacteria *Nitrosomonas cryotolerans* (ATCC 49181, Jones et al. 1988) and *Nitrosococcus oceanus* (ATCC 19707) were grown axenically in dark 2 l chemostat culture units (dilution rate,  $0.1 \text{ d}^{-1}$ ). The pH was adjusted automatically and maintained at  $7.8 \pm 0.1$  by the addition of sterile 5%  $\text{K}_2\text{CO}_3$ . The medium for ammonium oxidizers (Jones & Hood 1980) contained 12 mM of  $\text{NH}_4^+$  as  $(\text{NH}_4)_2\text{SO}_4$  at a salinity of 30‰ adjusted by the addition of Instant Ocean Synthetic Sea Salts (Aquarium Systems, Mentor, OH, USA).

The marine nitrite oxidizing bacteria *Nitrococcus mobilis* (ATCC 25380) and *Nitrobacter* sp. (Nb 297, provided by S. W. Watson) were grown in the same manner in the medium of Watson & Waterbury (1971). pH was adjusted automatically and maintained at  $7.5 \pm 0.5$  by injecting filter sterilized ( $0.2 \mu\text{m}$ )  $\text{CO}_2$ . The medium for nitrite oxidizers contained 30 mM of  $\text{NO}_2^-$  as  $\text{NaNO}_2$ . All cultures were constantly aerated, mixed with a magnetic stirring bar and kept at a constant temperature of  $25^\circ\text{C}$ .

**Preparation of standard cell suspension.** Cells (100 ml) were harvested by centrifugation ( $10\,000 \times g$ , 15 min,  $5^\circ\text{C}$ ) from chemostat units. The cell pellet was washed twice with  $0.2 \mu\text{m}$  filtered sea water (FSW), pH 7.8. The sea water used was low enough in nutrients that it did not have any consequence on the results (concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$  were below 20 nM). Cell densities were assessed with a Petroff-Hauser counting chamber and were made up to vary from  $1 \times 10^5$  to  $1 \times 10^7$  cells  $\text{ml}^{-1}$ , depending on the experiment.

**Description of illumination system.** A 1 kW xenon (short-arc) lamp (Optical Radiation Corp., Azusa, CA, USA) enclosed in an LH 151N lamp housing (Spectral Energy Corp., Hillsdale, NJ, USA) was used as a light source. This high-intensity light beam was focused through a GM-252 1/4 monochromator (Spectral Energy Corp.) into the sample compartment. The tunable GM-252 monochromator had adjustable slits to control entrance, height and width of the light beam. Entrance slit was set according to the light intensity required (0 to 0.6 mm): slit height was set at 6.5 mm and the exit slit was set to allow a 20 nm bandpass. Transmitted light quality and quantity were determined by wavelength scans using a 1/4 monochroma-

tor Model 77200 (Oriol Corp., Stratford, CT, USA) attached to a photodiode detection system Model 7072 (Oriol Corp.). The voltage signal from the detector was converted to radiometric units by using a 78250 Voltage Radiometry Board (Oriol Corp.) that interfaced with the PC-based detection system. The radiometric measurements were checked against potassium ferrioxalate actinometry (Hatchard & Parker 1956).

**Monochromatic illumination of cells.** Cylindrical quartz cuvettes (light path, 100 mm) were used to hold the volume of cell suspension to be irradiated. The total volume including cuvette (30 ml) and pump tubing (9 ml) was 39 ml. Considering this volume was kept under continuous flow ( $14 \text{ ml min}^{-1}$ ; Ismatec 4 channel pump), it was assumed that each cell was directly in the light path for only  $\frac{1}{3}$  of the total time of irradiation. The cuvettes were initially filled with FSW that contained the substrate at the desired concentration for the test. Once the cuvettes were filled, the pump was started and the outside reservoirs were filled to a constant level for all the experiments. The design allowed for the FSW (with substrate) to go first through a refrigerated circulator (Haake A81) that kept the entire system at  $20^\circ\text{C}$  during the irradiations, and then back into the sample compartment.

Once temperature was stable, a 0.2 ml portion of the standard cell suspension was inoculated into each of the 2 reservoirs (experimental and control). Therefore, the cell suspension to be irradiated consisted of FSW, the substrate of interest ( $\text{NH}_4^+$  or  $\text{NO}_2^-$ ) at the appropriate final concentrations (10 to 100  $\mu\text{M}$   $\text{NH}_4^+$  or 2 to 5  $\mu\text{M}$   $\text{NO}_2^-$ ) and the microorganisms ( $\text{NH}_4^+$  or  $\text{NO}_2^-$  oxidizers). No additional buffering agent was included since the low cell densities used in these experiments along with the short irradiation periods did not significantly alter the pH of the cell suspension. This was confirmed by measuring pH of the cell suspension before and after irradiation. After inoculation of the cuvettes, 2 min were allowed for obtaining an even distribution of the cells in the cuvette and sampling reservoir under the flow conditions used. The monochromator's exit slit was then opened to start the irradiation period. At this point, the time 0 ( $T_0$ ) samples were collected, and the timing for the total irradiation period was initiated. For the purpose of sampling at different time intervals, the system was kept open to the atmosphere, which also had the advantage of providing saturating  $\text{O}_2$  conditions and no accumulation of photochemically formed CO ( $<0.3 \text{ nM}$ ). The light intensities ranged from 5 to  $25 \text{ W m}^{-2}$ .

**Polychromatic illumination of cells. Sunlight:** Conditions of flow, temperature, and cell suspension composition were kept the same as in the laboratory set-up described above. The control cuvette and tubing were appropriately wrapped with aluminum foil while the

experimental cuvette and tubing were left exposed to sunlight. Light intensity was measured in this case with an IL 1700 research radiometer. Sunlight irradiance during the experiments ranged from 360 to 400 W m<sup>-2</sup> (400 to 1100 nm).

**Cool-white fluorescent irradiation:** For these experiments daylight (cool-white) fluorescent tubes (General Electric F15T8/CW) were used. Two tubes were placed diagonally opposing on each side of the chemostat bottle. The fluorescent lamps were connected to a 24 h timer, which permitted simulation of day-night cycles (i.e. 12:12 h light:dark; 8:16 h light:dark). These experiments were conducted after the chemostats had reached steady state (i.e. constant nitrite concentration, approx. 4 volume changes), but before visible cell attachment to the vessel walls occurred. Twenty ml of chemostat culture was harvested by centrifugation (10000 × *g*, 15 min, 5°C), washed twice with FSW and resuspended in 1 ml of FSW. From this cell suspension 10 µl was taken and diluted into 10 ml of distilled water to determine nitrite concentrations. The pH was also measured at the different sampling times.

**Action spectra.** The following conditions were used for action spectroscopy: light intensity 25 W m<sup>-2</sup>; final substrate concentration 100 µM NH<sub>4</sub><sup>+</sup> and 2 µM NO<sub>2</sub><sup>-</sup>; and cell density 10<sup>6</sup> cells ml<sup>-1</sup>. Irradiation times were selected on the basis of time course experiments (see below, 'Results') as 2 and 4 h for NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> oxidizers, respectively. Monochromatic irradiations were done in 25 nm increments that started with light of 300 nm and ended at 500 nm. Above 500 nm there was no detectable photoinhibition (see Fig. 2A, B).

**Other variables tested. Substrate concentration:** Final substrate concentrations of NH<sub>4</sub><sup>+</sup> or NO<sub>2</sub><sup>-</sup> were adjusted by adding an appropriate amount from 10 mM stock solutions of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> or NaNO<sub>2</sub>, respectively.

**Cell density:** Cell suspensions were made up to an appropriate cell density by increasing or decreasing the final resuspension volume with FSW, and confirming the preestimated cell number by microscopic counts.

**Cell product assay:** After 2 h of monochromatic irradiation 1 ml was collected from each reservoir (experimental and control) and was added to 60 ml serum bottles that contained 10 ml FSW, NO<sub>2</sub><sup>-</sup> (final concentration, 2 µM) and approximately 1 × 10<sup>6</sup> cells ml<sup>-1</sup> of actively growing nitrite oxidizing bacteria. Three duplicate sets were used. The control set received a 1 ml dose from the dark cuvette and remained wrapped in foil during the rest of the incubation. The other 2 sets received 1 ml samples from the irradiated cuvette. All serum bottles were allowed to incubate on a rotatory shaker at 150 rpm at 25°C for 4 h.

In addition, absorbance scans of cuvette contents were run before and after irradiations. A new peak or

shift from original absorption maxima was taken as an indication of an additional product being formed during illumination.

**Dose rate:** To test for reciprocity (dose × time), light fluences of 20 to 25 and 10 to 12 W m<sup>-2</sup> were used for 2 and 4 h, respectively.

**Analytical methods.** Nitrifying activity was assayed by NO<sub>2</sub><sup>-</sup> determinations using the spectrophotometric method of Bendschneider & Robinson (1952). Nitrite production was used as an index of ammonium oxidation, and nitrite disappearance as an indication of nitrite oxidation.

Replicate samples were run for each experiment. Samples were collected in duplicates, and the data points correspond to the mean of the duplicates. Correlation analyses (*r*<sup>2</sup>) and Student's *t*-tests were used to determine relationships between 2 variables and statistical significance, respectively.

## RESULTS

In order to choose appropriate conditions for action spectroscopy (photoresponse vs λ), it was necessary to first determine a time course of photoinactivation for NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> oxidation (Fig. 1). The 2 types of nitrifi-

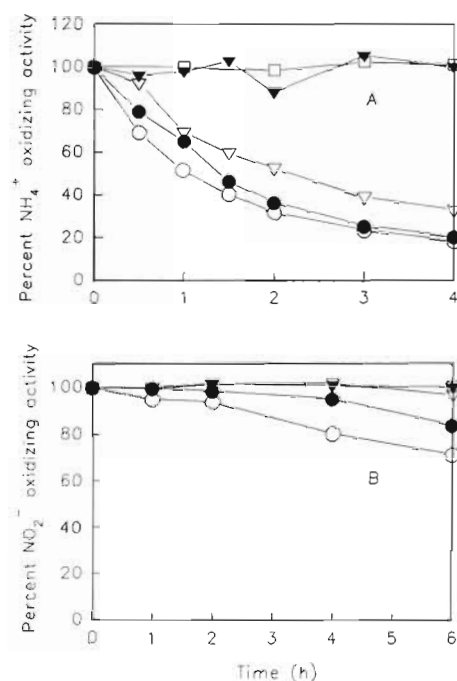


Fig. 1 Time course of photoinactivation. (A) *Nitrosomonas cryotolerans* after a total irradiation period of 4 h with 10 µM NH<sub>4</sub><sup>+</sup> (B) *Nitrobacter* sp. (25W30N) after 6 h with 2 µM NO<sub>2</sub><sup>-</sup>. Activities are expressed at 1 × 10<sup>6</sup> cells ml<sup>-1</sup>. Light intensity was 25 W m<sup>-2</sup>. (O) 300 nm; (●) 400 nm; (▽) 475 nm; (▼) 500 nm; (□) 600 nm.



fying bacteria ( $\text{NH}_4^+$  and  $\text{NO}_2^-$  oxidizers) exhibited different rates of photoinactivation. For instance, *Nitrosomonas cryotolerans* exhibited a noticeable decrease in activity, showing a 20 to 30% loss of activity after only 30 min of light exposure (Fig. 1A). Light sensitivity was more marked at the lower end of the spectrum (300 to 400 nm), though longer wavelengths were still effective during relatively short periods of exposure (e.g. 30% inhibition after 1 h of 475 nm irradiation). In contrast, *Nitrobacter* sp. required longer periods of illumination to show decreased activity (Fig. 1B). A significant reduction in the rates of nitrite oxidation ( $p < 0.05$ ) was observed only after a minimum of 4 h of irradiation with the shorter wavelengths (300 to 375 nm).

The increase of ammonium oxidation rates when substrate concentration was raised (from 10 to 100  $\mu\text{M}$   $\text{NH}_4^+$ ) enhanced light tolerance in ammonium oxidizers (Fig. 2A, B). For *Nitrosomonas cryotolerans*, an otherwise 60% inhibition (450 nm; Fig. 2A) could be reduced by more than half (450 nm; Fig. 2B) when  $\text{NH}_4^+$  concentration was increased. The same substrate photoprotection was not observed with *Nitrococcus mobilis* which did not significantly alter their light response ( $p > 0.05$ ; Fig. 2C, D), regardless of the substrate concentration employed (2 or 5  $\mu\text{M}$   $\text{NO}_2^-$ ). In the case of ammonium oxidizers, the only instance in which substrate protection did not apply was with the shortest wavelength tested (300 nm; Fig. 2B).

The total number of bacteria present could influence the light response among nitrifying bacteria differently. Light sensitivity by *Nitrosomonas cryotolerans* was not altered significantly ( $p > 0.05$ ) when the number of cells in the suspension was increased from  $10^4$  to  $10^6$  cells  $\text{ml}^{-1}$  (Fig. 2E, F). At most, a change from 12 to 28% activity was detected when using cell concentrations of  $10^4$  and  $10^6$  cells  $\text{ml}^{-1}$  (Fig. 2F), respectively.

In contrast, the nitrite oxidizer *Nitrobacter* sp. exhibited a statistically significant ( $p < 0.05$ ) light sensitivity at higher cell densities (Fig. 2G, H). Light with a wavelength of 425 nm that did not have an inhibitory effect became inhibitory whenever cell densities above  $10^6$  cells  $\text{ml}^{-1}$  were used (data not shown). Ammonium oxidizers did not exhibit the same behavior.

By adding conditioned medium (1 ml) from irradiated cultures to actively growing cultures of nitrite oxidizers it was possible to test for a cell product capable of inhibiting nitrite oxidizing activity, as summarized in Table 1. Cultures added with the conditioned media started accumulating nitrite, denoting decreased nitrite oxidation. In addition, if the irradiated sample was previously filtered (0.2  $\mu\text{m}$ ) the effect was not as evident. Absorbance scans of the light cuvette contents run before and after irradiations did not show a noticeable change. A shift in difference maxima would have been taken as an indication of a new product.

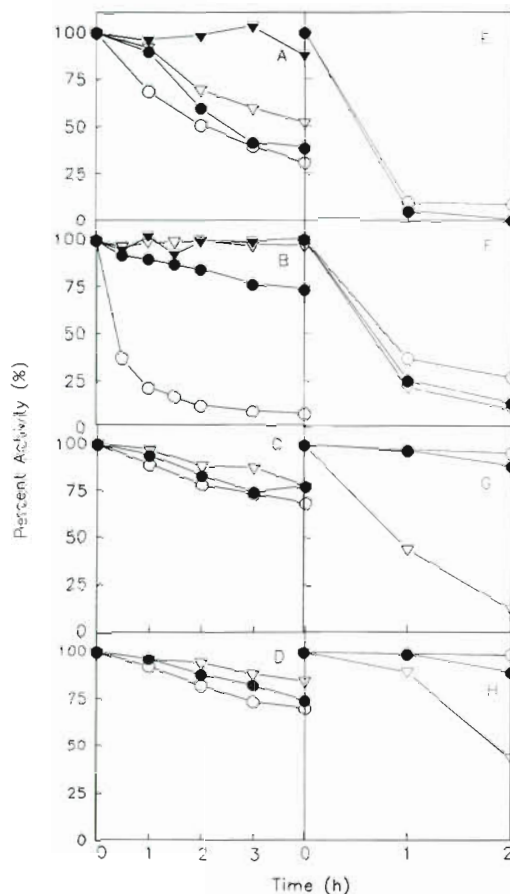


Fig. 2. Effect of substrate concentration and cell density on photoinactivation by different wavelengths of light. Substrate concentration: *Nitrosomonas cryotolerans*: (A) 10  $\mu\text{M}$   $\text{NH}_4^+$ , (B) 100  $\mu\text{M}$   $\text{NH}_4^+$ ; (O) 300 nm, (●) 450 nm, (▽) 475 nm, (▼) 500 nm. *Nitrococcus mobilis*: (C) 2  $\mu\text{M}$   $\text{NO}_2^-$ , (D) 5  $\mu\text{M}$   $\text{NO}_2^-$ ; (O) 300 nm, (●) 350 nm, (▽) 400 nm. *N. cryotolerans* with 100  $\mu\text{M}$   $\text{NH}_4^+$ : (E) 300 nm, (F) 450 nm; cell densities were (O)  $1 \times 10^4$  cells  $\text{ml}^{-1}$ , (●)  $1 \times 10^5$  cells  $\text{ml}^{-1}$ , (▽)  $1 \times 10^6$  cells  $\text{ml}^{-1}$ . *N. mobilis* with 2  $\mu\text{M}$   $\text{NO}_2^-$ : (G) 325 nm, (H) 375 nm; cell densities were (O)  $10^4$  cells  $\text{ml}^{-1}$ , (●)  $10^5$  cells  $\text{ml}^{-1}$ , (▽)  $10^6$  cells  $\text{ml}^{-1}$ . Activities are expressed at  $1 \times 10^6$  cells  $\text{ml}^{-1}$ . Light intensity was  $25 \text{ W m}^{-2}$ .

Table 1. Nitrite accumulation by actively growing *Nitrobacter* spp. in the presence of conditioned media (conditioned or spent medium was obtained after cells' activity took place for a period of 3 to 4 h)

Time (h)	Control	$[\text{NO}_2^-]$ , $\mu\text{M}$ 0.2 $\mu\text{m}$ filtered	Unfiltered
0	1.44 (100%)	1.44 (100%)	1.44 (100%)
1	1.19 (83%)	1.34 (93%)	1.31 (91%)
2	0.98 (68%)	1.23 (85%)	1.15 (80%)
3	0.86 (60%)	1.23 (85%)	1.02 (71%)
4	0.65 (45%)	1.12 (78%)	0.82 (57%)
5	0.57 (40%)	1.12 (78%)	0.78 (54%)

Photoinhibition was found to depend not only upon the quality but also the quantity of light. Light intensities as low as  $15 \text{ W m}^{-2}$  could cause a 50 to 70% inhibition of  $\text{NH}_4^+$  oxidizing activity after a 2 h exposure ( $p < 0.05$ ; Table 2). Higher doses ( $25 \text{ W m}^{-2}$ ) did not change the end result noticeably. It should be noted that the dose-dependent response was more dramatic at the shorter wavelengths (300 to 400 nm). The longer wavelength tested (500 nm) remained ineffective even at the highest dose used ( $25 \text{ W m}^{-2}$ ). Nitrite oxidizers showed negligible photoinactivation when subjected to monochromatic irradiations at a dose of  $25 \text{ W m}^{-2}$  (Table 2). Only near-UV light in conjunction with high cell densities (Fig. 2G, H) resulted in some photoinactivation.

The efficiency of photoinactivation changes, depending on the dose and rate of exposure. To test for reciprocity (dose  $\times$  time), light fluences of 20 to 25 and 10 to 12  $\text{W m}^{-2}$  were used for 2 and 4 h, respectively. *Nitrosomonas cryotolerans* results (Fig. 3) are shown as representative of the ammonium oxidizer response. Highest light sensitivities appear at the shorter wavelengths (300 to 400 nm), but the extent of light inhibition is reduced at all wavelengths in general (300 to 475 nm) whenever the lower dose ( $10 \text{ W m}^{-2}$ ) and the longest time of exposure (4 h) were used.

Nitrite oxidizers showed the opposite response (Fig. 3B). The lower dose ( $12 \text{ W m}^{-2}$ ) along with the longer time of exposure (4 h) increased their light sensitivity ( $p < 0.05$ ). The apparent lack of trend observed (lower dose = higher inhibition) is a result of the difficulty of using the same number of cells from experiment to experiment, and, as discussed above, cell density variation can alter significantly the expected phototolerance of nitrite oxidizers.

#### Monochromatic illumination: wavelength-dependent response

Action spectral analyses were conducted to determine the wavelength limits of the radiation causing photoinhibition.  $\text{NH}_4^+$  oxidizing activity was drastically decreased at the shorter wavelengths (300 to 375 nm). The activities recorded for *Nitrosomonas cryotolerans* as well as for *Nitrosococcus oceanus* at these wavelengths did not exceed 15%. For *N. cryotolerans* there was a gradual loss of light sensitivity (i.e. 55% inhibition at 450 nm, 32% inhibition at 475 nm, etc.) at the

Table 2. Influence of light intensity on nitrifying activity

	$\text{W m}^{-2}$	<i>N. cryotolerans</i>	<i>N. oceanus</i>	<i>Nitrobacter</i> sp.	<i>N. mobilis</i>
Sunlight <sup>a</sup>	400	1.2	6.25	83	33
Monochromatic <sup>b</sup>					
300 nm	5	30.8			
	15	31.47			
	25	22.1			
350 nm	5	64.86		98.8	
	15	32.89		100	
	25	32		97.5	
400 nm	5	79.2			
	15	34.61			
	25	47.12			
475 nm	5	87.91		96.58	
	15	52.11		88.59	
	25	39.28		99.71	

<sup>a</sup>Irradiation time was 2 h. Activities are expressed at  $1 \times 10^6$  cells  $\text{ml}^{-1}$   
<sup>b</sup>*N. cryotolerans* was irradiated for 2 h with  $10 \mu\text{M NH}_4^+$ . *Nitrobacter* sp. was irradiated for 4 h with  $2 \mu\text{M NO}_2^-$ . Activities are expressed at  $1 \times 10^6$  cells  $\text{ml}^{-1}$

longer wavelengths (400 to 475 nm). For *N. oceanus* there was a steady light inhibition up to 475 nm (80% inhibition) and a sharp drop in photosensitivity above 475 nm (Fig. 4A).

Overall  $\text{NO}_2^-$  oxidizing activity showed a remarkable phototolerance when compared to  $\text{NH}_4^+$  oxidizing

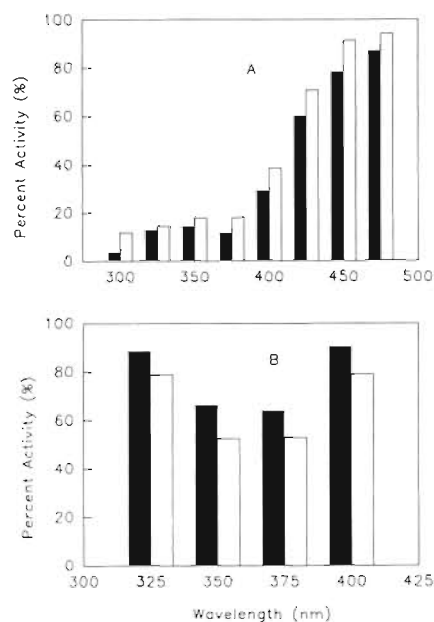


Fig. 3. Dose rate relationships: (A) *Nitrosomonas cryotolerans*, with  $100 \mu\text{M NH}_4^+$ ; (B) *Nitrosococcus mobilis*, with  $2 \mu\text{M NO}_2^-$ . Wavelengths used were from 300 to 475 nm in 25 nm increments. Activities are expressed at  $1 \times 10^6$  cells  $\text{ml}^{-1}$ . Solid bars: 2 h at 20 to 25  $\text{W m}^{-2}$ ; open bars: 4 h at 10 to 12  $\text{W m}^{-2}$

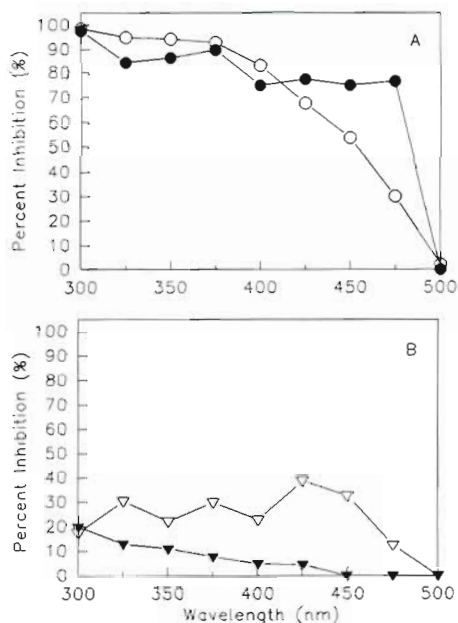


Fig. 4. Action spectra. (A) Ammonium oxidizing activity with  $100 \mu\text{M NH}_4^+$ . Irradiation time was 2 h. (B) Nitrite oxidizing activity with  $2 \mu\text{M NO}_2^-$ . Irradiation time was 4 h. Light intensity was  $25 \text{ W m}^{-2}$ . Activities are expressed at  $1 \times 10^6 \text{ cells ml}^{-1}$  (O) *Nitrosomonas cryotolerans*; (●) *Nitrosococcus oceanus*; (▽) *Nitrococcus mobilis*; (▼) *Nitrobacter sp.* (25W30N)

activity. The slight photoinactivation experienced by nitrite oxidizers (ca 15%) was limited to the shorter wavelengths (300 to 450 nm; Fig. 4B). Comparatively, *Nitrococcus mobilis* exhibited more photoinhibition (20 to 40%) and the same pattern as  $\text{NH}_4^+$  oxidizers: the shorter the wavelength, the greater the light inhibition. Yet, the reduction of  $\text{NO}_2^-$  oxidizing activity (<40%; Fig. 4B) is still significantly lower ( $p < 0.05$ ) than the decrease in  $\text{NH}_4^+$  oxidizing activity (<90%; Fig. 4A).

### Effect of polychromatic illumination

#### Cool-white fluorescent light

Because nitrite oxidizers were found to be insensitive to this type of light (Vanzella et al. 1989), only ammonium oxidizers were tested in this respect. Both ammonium oxidizers were examined by inflicting fluorescent illumination on the chemostat bottles in which they were growing. Illumination periods were of 8 or 12 h followed by 16 or 12 h dark periods, respectively. Both illumination treatments gave an immediate response. For instance, by sampling at hourly intervals, it was observed that within the first hour the pH in the chemostat increased, while the nitrite concentration dropped (Table 3). With the 8:16 h light:dark chemostat, pH increased from 7.85 to

Table 3. Effect of periodic illumination on *Nitrosomonas cryotolerans* (chemostat)

Time (h)	8:16 h light:dark		12:12 h light:dark	
	pH	$\text{NO}_2^-$ , $\mu\text{M}$	pH	$\text{NO}_2^-$ , $\mu\text{M}$
1	7.85	4.46	7.84	4.5
2	7.93	3.76	7.86	4.37
3	8.04	3.51	7.94	3.5
4	8.12	3.76	8.06	3.3
5	8.14	2.71	8.13	2.8
6	8.17	2.99	8.22	2.7
7	8.24	2.80	8.23	2.1
8	8.27	1.46	8.25	1.7
9			8.27	1.0
10			8.30	0.7
11			8.35	0.5
12			8.35	0.58

8.3, while nitrite decreased from 4.5 to 1.5  $\mu\text{M}$ , denoting the decrease in activity experienced upon fluorescent illumination.

#### Sunlight

The monochromatic and fluorescent light sources used in this study show considerable qualitative and quantitative differences in their spectra as compared to sunlight. No attempt was made to filter sunlight or to decrease light intensity. Instead, the light dose received by all the species tested was kept almost uniform, such that the integrated light fluence was within the 360 to 400  $\text{W m}^{-2}$  range. An almost complete inhibition was detected within the first 30 min, in the case of ammonium oxidizers (20% remaining activity). Both *Nitrosomonas cryotolerans* and *Nitrosococcus oceanus* lost up to 90% of their  $\text{NH}_4^+$  oxidizing activity after 2 h of sunlight exposure (Table 2). Nitrite oxidizers appeared somewhat sunlight-resistant, because their activities did not decrease to the extent of those of ammonium oxidizers. Thus, sunlight experiments served to verify the observed near-UV sensitivity common to both types of nitrifiers.

### DISCUSSION

This study confirmed the fact that marine ammonium and nitrite oxidizers can be photoinactivated, yet it differs from the previous reports in as much as which microorganisms were more light-sensitive. The use of natural populations as opposed to pure cultures, the species employed, and the light sources and intensities used can help to explain the difference in results with previous investigations. In earlier studies with either soil (Müller-Neuglück & Engel 1961, Schön & Engel

1962) or aquatic nitrifiers (Olson 1981, Yoshioka & Saijo 1984, Diab & Shilo 1988),  $\text{NO}_2^-$  oxidizers were described as more light-sensitive than  $\text{NH}_4^+$  oxidizers. In this case, and at the various wavelengths tested (300 through 500 nm), both of the  $\text{NH}_4^+$  oxidizers tested (*Nitrosomonas cryotolerans* and *Nitrosococcus oceanus*) exhibited a greater light sensitivity than the nitrite oxidizers (*Nitrococcus mobilis* and *Nitrobacter* sp.). These types of results agree with the previous work of Vanzella et al. (1989).

Each species varied in their individual light responses (Fig 4A, B); for instance, *Nitrococcus mobilis* was more light-sensitive than *Nitrobacter* sp., and among the ammonium oxidizers, *Nitrosococcus oceanus* was more photosensitive than *Nitrosomonas cryotolerans*. Strain variability is recognized in instances such as substrate and product inhibition (Boon & Laudelot 1962, Focht & Verstraete 1977), effect of nitrification inhibitors (Belser & Schmidt 1981, Jones & Morita 1984), resistance to high oxygen concentrations (Gunderson 1966), etc. Thus, it is not surprising that light response can vary markedly between different species and different genera. Natural ecosystems show a coexistence of different species of nitrifying bacteria (Fliermans et al. 1974, Belser & Schmidt 1978a, b) that will more than likely exhibit different degrees of photosensitivity, but whether this is significant in natural environments remains to be assessed.

Action spectroscopy revealed a widespread photosensitivity in the near-UV (300 to 375 nm) region for nitrifying bacteria. Nevertheless, the relative phototolerance of nitrite oxidizers was evidenced even at these relatively short wavelengths. Important naturally occurring chromophores in this region of the spectrum may be either 4-thiouridine, pyridoxine compounds, nicotinamide adenine dinucleotide (NADH), or electron transport molecules. 4-thiouridine ( $^4\text{Srd}$ ) is an unusual DNA base which can extend the high far-UV absorption of transfer RNA into the near-UV region, with a peak at 340 nm provoking a variety of sublethal biological effects (Jagger 1983). Pyridoxine compounds act as prosthetic groups or coenzymes, and show strong absorption throughout the near-UV region, with peaks at 325 and 388 nm (Jagger 1981). NADH in its reduced form absorbs light best at 340 nm (Jagger 1981). Quinones and flavins are among the suggested candidate electron transport molecules (Epel 1973). The inhibition continuum observed in the near-UV region could be the result of absorption by any of the above or of the summation of the individual absorption properties of the above. The relevance of near-UV impacting marine environments is limited to the surface of oceans; therefore, the complete elucidation of the phototarget in the near-UV region was not pursued.

The visible region examined in this work (400 to 500 nm) was where most of the differential photosensitivity among nitrifiers was exhibited. This type of light did not disturb nitrite oxidizing activity considerably (ca <15%; Fig 4B); yet, photoinhibition of up to 80% was detected for ammonium oxidizing activity (Fig. 4A). Action spectra of intact organisms constitute a good start for the evaluation of biological responsiveness to monochromatic (i.e. narrowband) irradiation. Yet, it can be argued that the effect of polychromatic (i.e. broadband) irradiation is of more ecological relevance. Broadband irradiation more closely approximates the total influence, both beneficial and detrimental, of all wavelengths present in natural sunlight. The similarities (intensities) and differences (wavebands) of the various types of light (monochromatic, polychromatic) can be considered an advantage, since results can be compared and contrasted. For instance, the blue sensitivity of ammonium oxidizers was corroborated when broadband irradiations were performed using cool-white fluorescent light. Similarly, nitrite oxidizers were confirmed as blue light insensitive, since fluorescent illumination did not affect them. The photosensitivity of both types of nitrifying bacteria to near-UV light was corroborated when these organisms were subjected to sunlight irradiations.

In this work nitrifying bacteria also showed opposing responses to other factors besides light. One of the factors studied was that of substrate protection.  $\text{NH}_4^+$  oxidizers became less photosensitive in the presence of higher  $\text{NH}_4^+$  concentrations, while  $\text{NO}_2^-$  oxidizers did not significantly change their light response if nitrite concentration was increased.

At substrate concentrations of 2 and 100  $\mu\text{M}$  for  $\text{NO}_2^-$  and  $\text{NH}_4^+$ , respectively, it is expected that both nitrite and ammonium can enter the cells by passive diffusion. However, ammonium oxidizers in marine environments experience only submicromolar concentrations of  $\text{NH}_4^+$  and may survive in this habitat due to the highly efficient enzyme systems and adaptations that pelagic marine bacteria possess (Azam & Hodson 1981, Jones & Morita 1985). At this point, there are at least 3 possibilities: it can be argued that such highly specialized marine bacteria may have lost their low substrate affinity by maintenance in pure culture; that the detrimental effects of light affect  $\text{NH}_4^+$  transport mechanisms, if any; or that rapidly growing cells (i.e. high nitrification rates) better tolerate the constraints that light imposes on them. Assuming that repairing photoimpact is an energy-dependent process, those cells with higher nitrification rates would more efficiently cope with the damage caused by light. Perhaps, at low substrate concentrations, one or a combination of the abovementioned factors could have intervened on the activity measurements and made it

appear as if excess substrate had a photoprotective effect.

Nitrite oxidizers are considered versatile in many respects. They are known for their heterotrophy (Bock 1976), mixotrophy (Smith & Hoare 1968), heterogeneity among species (Navarro et al. 1992), anaerobic growth by dissimilatory nitrate reduction (Freitag et al. 1987, Bock et al. 1988, Freitag & Bock 1990), as well as alternate respiratory chains (Bock et al. 1986, Nomoto et al. 1993). The inherent metabolic diversity of nitrite oxidizers may be the key to their phototolerance. For instance, if light affected 1 electron mediator in the electron transport system (ETS), there is the possibility that nitrite oxidizers could switch to an alternate respiratory component and therefore withstand illumination at lower substrate concentrations much better than could their counterpart (ammonium oxidizers). More work concerning regulation and control of substrate oxidation could clarify this matter.

Cell density is another factor that can modify light response by nitrifying bacteria. At higher cell densities there was a considerable decrease in activity for nitrite oxidizers ( $p < 0.05$ ), but not for ammonium oxidizers. The contrasting results ( $\text{NH}_4^+$  oxidizers vs  $\text{NO}_2^-$  oxidizers) were not the product of pH shifts. The nature of these short-term (2 to 4 h) activity measurements did not allow for a change in pH, outside the effective pH range for autotrophic nitrification (pH 7 to 8). A maximum drop of 0.3 pH units was measured at the end of the incubation period, when cell densities of  $10^6$  cells  $\text{ml}^{-1}$  were used.  $\text{O}_2$  limitation was also not considered to be significant. Although the  $K_m$  for oxygen consumption is high among nitrifying bacteria (0.3 to 1.0 mg  $\text{O}_2$   $\text{ml}^{-1}$ ; Boon & Laudelot 1962, Loveless & Painter 1968),  $\text{O}_2$  was not limiting as evidenced by nitrite oxidation rates observed in controls.

A simple cell product test showed a relationship between adding conditioned media and greater photosensitivity by nitrite oxidizers. If it were only an end product causing the loss of nitrite oxidation, it is expected that cultures taken from both dark as well as irradiated cell suspensions would (at the same cell density) cause the same decrease in activity, but this was not the case. Thus, there is either an 'additional' product that is produced by the irradiated cells, or more of the end product is made under continuous illumination. The nature of the cell product was not investigated. Boon & Laudelot (1962) found a non-competitive product inhibition effected by nitrate, which at this point can be taken as a plausible explanation. In addition to the accumulation of nitrate as an end product, there is the potential for concurrent photochemical formation of nitrate (Zafiriou & True 1979) with the shortest wavelength used. These findings may also explain

previous reports (Müller-Neuglück & Engel 1961, Bock 1965, Olson 1981, Yoshioka & Saijo 1984, Diab & Shilo 1988) where cell densities were not considered an important variable and resulted in an increased photosensitivity of nitrite oxidizers as compared to ammonium oxidizers.

Photoinhibition depended on both the quality and the quantity of light. In this work, emphasis was placed on the action of wavelengths in the near-UV (300 to 400 nm) to the visible (400 to 500 nm) region, and not in far-UV (190 to 280 nm) since all cell types are susceptible to far-UV, and microorganisms under natural conditions are not subjected to these shorter wavelengths. A dose-dependent response was observed that demanded higher fluences to achieve the same photoinactivation at the longer wavelengths. This can be easily explained in terms of differential targets for the various types of light. For instance, far-UV can alter proteins and nucleic acids, which do not absorb significantly at longer wavelengths (i.e. 300 to 500 nm). Considering the vital role of DNA and proteins, it is expected that at longer wavelengths (>300 nm) the fluences required to produce biological effects, other than mutation and killing, will be much higher (Eisenstark 1971, Epel 1973, Jagger 1983).

Another critical condition that concerns photoprocesses is the biological dose to affect them; the dose being the product of irradiance and the length of exposure time. It was determined that reciprocity of time and dose did not apply in any of the cases, with either ammonium or nitrite oxidizers. Yet, even in this aspect both types of nitrifiers also differed. In the case of ammonium oxidizers, the short-time/high-intensity (2 h at 20 to 25  $\text{W m}^{-2}$ ) experiments showed a more drastic photoinactivation than those with long-time/low-intensity (4 h at 10 to 12  $\text{W m}^{-2}$ ). Presumably, as far as ammonium oxidizers are concerned, the lack of reciprocity may involve the time rates and spectral responses of their repair mechanisms in relation to the time rates and phototargets of the damaging radiation.

Nitrite oxidizers do not exhibit the same behavior: long-time/low-intensity exposure had a greater photoeffect than the short-time/high-intensity exposure. Another incongruity was that longer wavelengths were sometimes more effective than shorter ones (Fig. 3B). Part of the inconsistency of these results may have arisen from the use of different cell batches; the inability to keep the exact same number of cells from experiment to experiment, and the abovementioned cells' product inhibition. That longer exposure times favor photoinactivation represents a good line of evidence for the cell product argument, since its concentration increases with time.

There is ample laboratory evidence concerning photoinhibition of nitrification. However, the ability to



accurately and unambiguously predict light consequences in aquatic (freshwater/marine) habitats remains elusive. Difficulties in quantitatively extrapolating laboratory findings to natural populations include the complexity of the spectral characteristics of water and/or water movements, and the possibility that natural communities might adapt through selection of resistant types. For instance, Horrigan & Springer (1990) reported reduced sensitivity to light by ammonium oxidizers from shallow estuarine environments as compared to marine isolates. What cannot be distinguished from their data is whether the estuarine bacteria are intrinsically less sensitive to light than marine bacteria, or if higher nitrification rates were favored by the increased turbidity (i.e. decreased light penetration) this habitat may offer.

In any event, the assorted light responses described in this work suggest that when studying nitrification in a particular environment, it would be ideal to determine nitrification activity and diversity. That nitrification in nature proceeds under a range of conditions, even those intolerable for pure cultures, may be an indication of how the adaptive capabilities of nitrifying bacteria may be underestimated. These data indicate that photoeffect on nitrification can be modified if 1 or more of the variables cited above were changed. More work dealing with the different sensitivities of  $\text{NH}_4^+$  and  $\text{NO}_2^+$  oxidation would give more insight into the interrelationships of both groups and the consequences to the overall process.

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